

(19)



Europäisches Patentamt

European Patent Office

Office européen des brevets



(11) Publication number:

0 642 799 A1

(12)

EUROPEAN PATENT APPLICATION(21) Application number: **94113388.6**(51) Int. Cl.⁸: **A61K 47/48**(22) Date of filing: **26.08.94**(30) Priority: **09.09.93 EP 93114475**(43) Date of publication of application:
15.03.95 Bulletin 95/11(94) Designated Contracting States:
**AT BE CH DE DK ES FR GB GR IE IT LI LU NL
PT SE**(71) Applicant: **BEHRINGWERKE
Aktiengesellschaft
Postfach 1140
D-35001 Marburg (DE)
Applicant: LABORATOIRES HOECHST S.A.
Tour Roussel Nobel, Cédex 3
F-92080 Paris La Défense (FR)**(72) Inventor: **Bosslet, Dr. Klaus
An der Haustatt 64
D-35007 Marburg (DE)
Inventor: Czech, Dr. Jörg
Kreutzackerweg 2A
D-35041 Marburg (DE)
Inventor: Hoffmann, Dr. Dieter
Feuerdornweg 123
D-35041 Marburg (DE)
Inventor: Tillequin, Prof. François
70, rue de l'Amiral Mouches
F-75013 Paris (FR)
Inventor: Florent, Dr. Jean-Claude**

**23 rue des Causus
F-91940 Les Ulis (FR)
Inventor: Azoulay, Dr. Michel
16 rue du Regard
F-75006 Paris (FR)
Inventor: Monneret, Dr. Claude
9 avenue L'amoricière
F-75012 Paris (FR)
Inventor: Jacquesy, Prof. Jean-Claude
46 rue du Planty
F-86360 Buxerolles (FR)
Inventor: Gesson, Prof. Jean-Pierre
La Germonière-Montassie
F-86360 Chasseneuil du Poitou (FR)
Inventor: Koch, Prof. Michel
116 Elysées 2
F-78170 La Celle Saint Cloud (FR)
Inventor: Vasella, Prof. Andrea
Langackerstrasse 7
CH-8057 Zürich (CH)
Inventor: Hoos, Roland
Affolternstrasse 107
CH-8050 Zürich (CH)**

(74) Representative: **Lauppe, Hans Friedrich et al
Behringwerke AG
Abt. Patente & Verträge,
Postfach 1140
D-35001 Marburg (DE)**

(54) Improved Prodrugs for enzyme mediated activation.

(57) Enzymatically cleavable prodrugs with reduced Michaelis-Menten constant (K_m) are described.**EP 0 642 799 A1**

This invention refers to enzymatically cleavable prodrugs with reduced Michaelis-Menten constant (K_m).

A prodrug may be defined as a chemical which is non-toxic and pharmacodynamically inert, but which can be transformed in vivo to a pharmacologically active drug.

The invention refers to the field of drug-targeting, which deals with site-specific delivery of drugs in vivo. Site-specific delivery preferably increases the selectivity of drugs and reduces their undesirable side effects.

One potential approach to achieve a site-specific delivery consists in applying nontoxic prodrugs which can be site-specifically activated to cytotoxic drugs using prelocalized prodrug cleaving catalysts like enzymes, muteins derived from enzymes, catalytic antibodies, antibody enzyme conjugates or fusion proteins.

This approach combines the advantage of drug delivery via prodrugs (i.e. increased stability, adjusted solubility, improved route of administration, more favourable distribution, improved pharmacokinetics, bypassing resistance; T.A. Connors, *Xenobiotica* 16, 975-988, 1986) with the preferential tumour specific activation mediated by a catalytic principle. The use of exogenous enzymes or polyclonal antibody enzyme conjugates for prodrug activation was pioneered by Graffi (*Deutsche Offenlegungsschrift* 22 12 014), and Philpott et al. (*J. Immunol.* 111, 921, 1973).

More recently the original teaching from Graffi and Philpott was exemplified and improved by the use of monoclonal antibody enzyme conjugates as prodrug activating catalysts (Bagshawe et al., *Brit. J. Cancer*, 58, 700, 1988; Senter et al., *Bioconjugate Chem.* 4, 3-9, 1993) or fusion proteins (Bosslet et al., *Brit. J. Cancer*, 65, 234-238, 1992; Goshorn et al., *Cancer Res.* 53, 2123-2127, 1993).

Despite these improvements, the systems described so far have some major disadvantages for clinical applications:

a) monoclonal antibody enzyme conjugates produced by chemical coupling have as a major drawback a strong immunogenicity in man due to the xenogenic origin of the antibody moiety and the enzyme (Bagshawe et al., *Disease Markers* 9: 233-238, 1991). As a consequence of this high immunogenicity repetitive applications in man are possible only to a very limited extent;

b) fusion proteins consisting of non-humanised binding moieties and xenogenic enzymes produced by recombinant DNA technology will be immunogenic in man as well with disadvantages comparable to monoclonal antibody enzyme conjugates, if repetitive applications are needed;

c) fusion proteins consisting of humanised binding moieties and human enzymes will probably not be very immunogenic in man most probably allowing repetitive treatment cycles in man. Nevertheless, the two major disadvantages of human fusion proteins are the possibly lower turnover rate (V_{max}) of the human enzyme moiety as well as the possibly higher prodrug (substrate) concentration needed to obtain significant catalysis in comparison to xenogenic enzymes having a high turnover rate and a low Michaelis-Menten constant (K_m).

This limitation of human fusion proteins (low V_{max} and high K_m) given by the intrinsic nature of the human enzyme moiety can be overcome by state of the art methodology only to a very limited extent (factor 4) by random mutagenesis in the active site of the enzyme (Munir et al., *PNAS USA* 90:4012-4016, 1993).

Surprisingly, it has been found that the limitation by a high K_m , an intrinsic property of most human enzymes applicable for in vivo prodrug activation, can be overcome by novel prodrugs.

These prodrugs have the formula I,

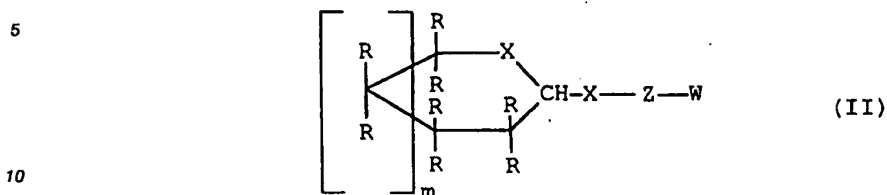
S-Z-W (I)

wherein W means a pharmacologically active substance, Z stands for a self-immolative spacer or a bond and S is a moiety such that the S-Z bond is enzymatically cleaved at an at least 2-fold lower Michaelis-Menten constant compared to the natural enzyme substrate.

The prodrugs of the invention have the common characteristic to be cleaved by enzymes at significantly lower molar prodrug concentration as the natural or standard substrates used for enzymatic analysis or appropriate state of the art prodrugs (WO 92/19639). They are therefore named K_m -reduced prodrugs.

The prodrugs of the invention have as another common characteristic a modified competitive enzyme activity inhibitor (S) as a crucial structural component which can be linked directly or via a spacer moiety (Z) to the pharmacologically active substance (W). Preferably the spacer is self-immolative generating the pharmacologically active substance after enzymatic cleavage of the S-Z bond. A self-immolative spacer is defined as a moiety which is bound through two bonds to two molecules and which eliminates itself from the second molecule if the bond to the first molecule is cleaved.

The preferred Km-reduced prodrugs are substrates for human glycosidases and have the general formula II:



wherein

R may be independent from each other

15 H, OH, F, NH₂, COOH, CH₂-COOH, CHOH-COOH, PO₃H₂, CH₂-PO₃H₂ or CHOH-PO₃H₂,

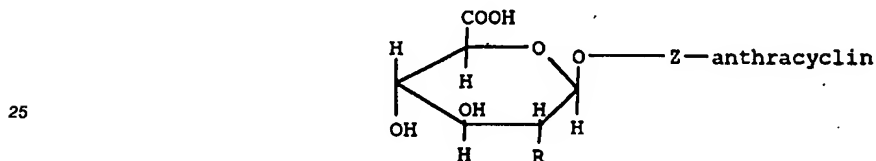
X may be NH, O or S,

m may be 0 or 1,

Z stands for a self-immolative spacer or a bond and

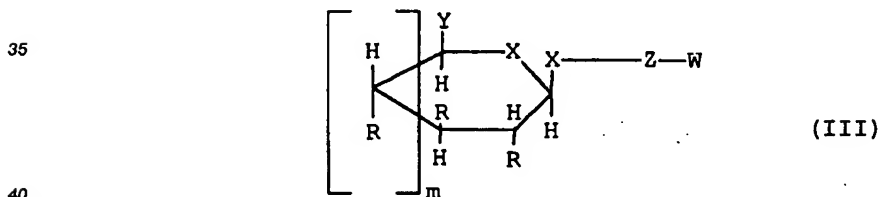
W means a pharmacologically active substance.

20 Not included are β-D-glucuronide-Z-anthracyclin compounds:



with R = OH, NH₂.

30 Especially preferred are Km-reduced prodrugs which are substrates for β-glucuronidase and have the general formula III:



wherein

Y may be COOH, CH₂-COOH, CHOH-COOH, PO₃H₂, CH₂PO₃H₂ or CHOH-PO₃H₂,

X may be NH, O or S,

45 R may be independent from each other F, NH₂, H or OH,

m may be 0 or 1,

Z stands for a bond or a self-immolative spacer preferentially a moiety with the formula



wherein

V is an aromate or a hetero aromate or an aliphate with conjugated double bonds or an amino acid residue which cycles after cleavage of the glycosyl residue, preferentially with 5-20 carbon atoms and 0-4 hetero atoms, wherein hetero atom means N, O or S, substituted with

55 R being independently from each other H, methyl, methoxy, carboxy, methoxycarbonyl, CN, hydroxy, nitro, fluor, chlor, brom, sulfonyl, sulfonamid or sulfon (C₁₋₄)-alkylamid and

p 0 or 1

n an integer of 0 to 25, preferentially 1 or 2

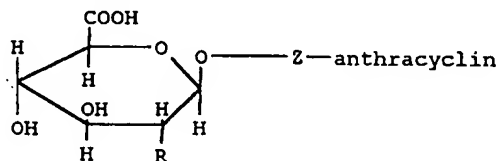
X O, NH, methylenoxy, methylenamino or methylen (C₁₋₄)-alkylamino,

Y O or NH

and

W means a pharmacologically active substance preferentially an anthracycline such as doxorubicin, 4'-epi-doxorubicin, 4- or 4'-desoxy-doxorubicin, or an etoposide, N-bis-(2-chlorethyl)-4-hydrox-
 5 yaniline, 4-hydroxycyclophosphamide, vindesine, vinblastine, vincristine, terfenadine, terbutaline, fenoterol, salbutamol, muscarine, oxyphenbutazone, salicylic acid, p-aminosalicylic acid, 5-fluorouracil, 5-fluorocytidine, 5-fluorouridine, methotrexate, diclofenac, flufenamic-acid, 4-methylaminophenazone, theophylline, nifedipine, mitomycin C, mitoxantrone, camptothecine, m-
 10 AMSA, taxol, nocodaxol, colchicine, cyclophosphamide, rachelmycin, cisplatin, melphalan, bleomycin, nitrogen-mustard, phosphoramidate-mustard, quercetin, genistein, erbstatin, tyrphostin, rohitukine-derivative ((-)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-on; EP 89119710.5), retinoic acid, butyric acid, phorbol ester, DMSO, ac-lacinomycin, progesterone, buserelin, tamoxifen, mifepristone, onapristone, N-(4-aminobutyl)-5-chloro-2-naphtalen-sulfonamide, pyridinyloxazol-2-one, quinolyl-, isoquinolyloxazolone-2-one, staurosporine, ethanolamine, verapamil, forskolin, 1,9-dideoxyforskolin, quinine, quinidine, reser-
 15 pine, 18-O-(3,5-dimethoxy-4-hydroxybenzoyl)-reserpate, lonidamine, buthionine sulfoximine, diethyldithiocarbamate, cyclosporine A, azathioprine, chlorambucil, N-(4-trifluormethyl)-phenyl-2-cyano-3-hydroxy-croton-acid-amide (WO 91/17748), 15-deoxyspergualin, FK 506, ibuprofen, indomethacin, aspirin, sulfasalazine, penicillamine, chloroquine, dexamethasone, prednisolone, lidocaine, propafenone, procaine, mefonamic acid, paracetamol, 4-aminophenazone, muskosine, orciprenaline, isoprenaline, amiloride, p-nitrophenylguanidinobenzoat or their derivatives additionally substituted with one or more hydroxy-, amino- or iminogroups, linked through a hydroxy-, amino- or imino group to Z.

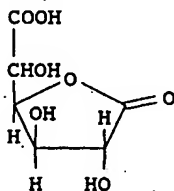
Not included are β -D-glucuronide-Z-anthracyclin compounds:



with R = OH, NH₂.

Enzyme in this application may also mean a catalytic antibody. The compounds described herein can be prepared by prior art methods.

Km-reduced prodrugs, selective for human β -glucuronidase, are described in the following sections. Prodrug A (example 1) may be looked upon as derived from the competitive β -glucuronidase inhibitor saccharolactone:



Example 1:

Prodrug A, B, C:

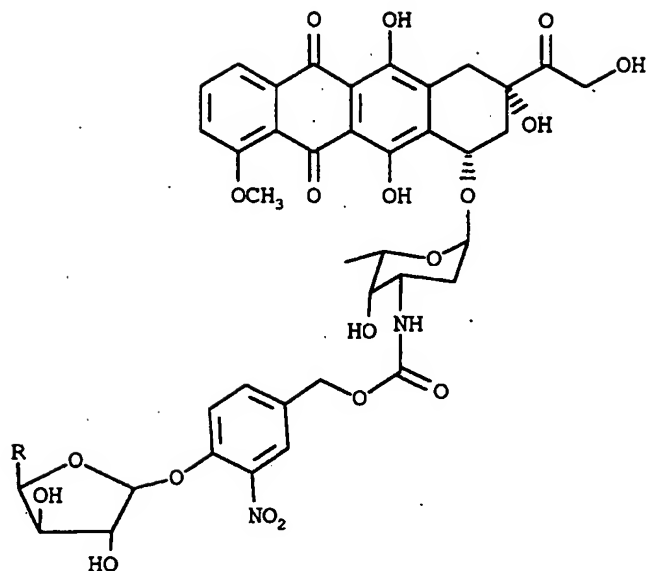
5

10

15

20

25

Prodrug A: R = CHOH-COOH Prodrug B: R = $\text{CH}_2\text{-COOH}$ 30 Prodrug C: R = COOH

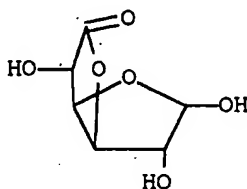
Experimental procedure for prodrug A:

Preparation of 1,2,5-tri-O-acetyl-aldehydo-D-glucurono-3,6-lactone (compound 7).

35

40

45



Compound 5

50

3,6-Glucarolactone (compound 5) (45 g) was slowly added to a cooled ($0-5^\circ\text{C}$) mixture of dry pyridine (225 ml) and Ac_2O (185 ml). The internal temperature was maintained at 5°C during the addition and after all the lactone has been dissolved, the reaction mixture was allowed to be stirred for additional 2 hours. The colourless solution was then poured into 3 liters of a mixture of water and crushed ice and vigorously stirred for approximately 3 hours. The precipitate was collected and washed with water, and after drying a solid was isolated which contains 70 g of a mixture of α and β tri-O-acetyl-glucuronolactone (compound 7). This mixture was directly used for the next step.

55

Preparation of 2,5-di-O-acetyl- α -D-glucurono-3,6-lactone- α -furanosyl bromide (compound 8).

Titanium bromide (16.6 g, 45 mmol) was added to a stirred solution of compound 7 (70 g, 23.3 mmol) in dichloromethane (200 ml) maintained in the dark and under nitrogen atmosphere. After stirring overnight, additional TiBr_4 was added (8.3, 22 mmol). After 24 additional hours, the reaction mixture was diluted with dichloromethane (150 ml) and the organic solution poured into crushed ice water. The organic layer was separated, washed with water, dried and evaporated under reduced pressure. This gave compound 8 (65 g) pure enough for the next step.

Preparation of (2-nitro-4-formylphenyl)-2,5-di-O-acetyl- β -D-glucurono-3,6-lactone furanoside (compound 9).

It was prepared from compound 8 (15 g, 50 mmol) and from 4-hydroxy-3-nitrobenzaldehyde according to the procedure already described in WO 92/ 19639. This afforded 12 g (61.6 %) of compound 9.

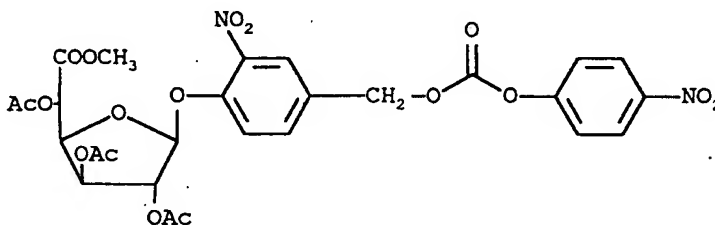
Preparation of (2-nitro-4-formylphenyl)-2,3,5-tri-O-acetyl- β -D-glucuronate (compound 1a).

To a solution of solid sodium hydroxide (50 mg) in methanol (125 ml), compound 9 (10 g) was added. The solution was stirred at room temperature for 4 h and evaporated under reduced pressure. This resulted in a crude mixture which was immediately dissolved in anhydrous pyridine (50 ml). After cooling to 0 ° C, acetic anhydride (40 ml) was added and the reaction mixture was subsequently stirred for additional 18 h. Extraction with dichloromethane followed by Usual work-up resulted in 6.6 g of compound 1a (65 % overall yield).

Preparation of (2-nitro-4-hydroxyethylphenyl)-2,3,5-tri-O-acetyl- β -D-glucuronate (compound 2a).

It was prepared by sodium borohydride reduction of compound 1a (6 g) according to the procedure already described in the WO 92/19639. This yielded 5.6 g (95 %) of compound 2a.

Preparation of 4-(2,3,5-tri-O-acetyl- β -D-methylglucuronofuranosyl)-3-nitro-p-nitrobenzoxycarbonate (compound 3a).

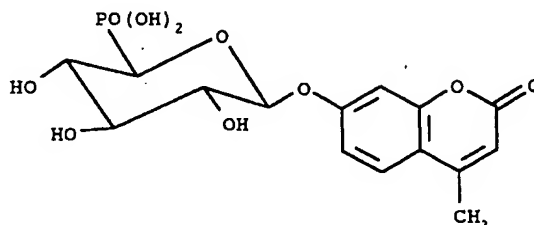


Compound 3a

It was prepared coupling of compound 2a (6 g) with 4-nitrophenyl chloroformate (yield 75 %) according to the procedure already described in the WO 92/19639.

Preparation of prodrug A:

Prodrug A was prepared from compound 3a and doxorubicin (yield 83 %) followed by treatment with sodium methoxide in methanol and then sodium hydroxide.

Example 2:**Preparation of 4-methylumbelliferyl (5R)-5-phosphonyl- β -D-xylopyranoside (16):**

Allyl 6-*O*-trityl- α -D-glucopyranoside (2). A solution of allyl α -D-glucopyranoside (1) (prepared according to R.E. Wing, J.N. BeMiller, Carbohydr. Res., 1969, 10, 441) (12.5 g, 56.8 mmol) and triphenylmethyl chloride (20.0g, 71.7 mmol) in dry pyridine (120 ml) was stirred at r.t. for 12 h, and at 60 ° for 1 h. After the addition of triphenylmethyl chloride (12.0 g, 43.0 mmol), the solution was stirred at 60 ° until all starting material has disappeared (3-4 h). H₂O (120 ml) was added to the still warm solution. Extraction with EtOAc, extraction of the combined org. layers with 1 M aq. H₂SO₄ and brine, evaporation of the organic layer and FC (400 g SiO₂, toluene/acctone 2:1 → toluene/acetone 1:1) gave 23.3 g (90 %) of 2. Grey glassy solid.

Allyl 2,3,4-tri-*O*-benzyl-6-*O*-trityl- α -D-glucopyranoside (3). A solution of 2 (15.9 g, 34.3 mmol) in dry THF (390 ml) was treated with a suspension of NaH (6.9 g, ca. 150 mmol) at r.t. for 10 min. BnBr (25.0 ml, 211 mmol) and Bu₄NI (1.9 g, 5.1 mmol) were added. The solution was heated under reflux until TLC indicated completion of the reaction (ca. 12 h). Et₂O was added, and the solution was filtered through silica. The filtrate was evaporated, and the residue was subjected to FC (600 g SiO₂, Et₂O/hexane 1:9 → Et₂O) to give 21.4 g (85%) of 3. R_f (EtOAc/hexane 1:4) 0.36. ¹³C-NMR (75 MHz, C₆D₆): 63.46 (t); 68.49 (t); 71.33 (d); 72.96 (t); 75.12 (t); 75.74 (t); 78.93 (d); 81.40 (d); 82.85 (d); 86.95 (s); 96.44 (d); 117.21 (t); 127.29-129.32 (several d); 134.73 (d); 139.11 (s); 139.32 (s); 139.89 (s); 144.78 (s, triple intensity).

Allyl 2,3,4-tri-*O*-benzyl- α -D-glucopyranoside (4). A solution of BF₃·OEt₂ (5.0 ml, 39.8 mmol) in MeCN (90 ml) was added dropwise to a cooled (0 °) solution of 3 (13.4 g, 18.3 mmol) and Et₃SiH (14.5 ml, 91.5 mmol) in dry CH₂Cl₂ (150 ml). After 10 min., a sat. aq. solution of NaHCO₃ (100 ml) and H₂O (200 ml) were added. The mixture was shaken vigorously, the aq. layer was extracted with CH₂Cl₂, the combined organic layers were extracted with brine, dried (MgSO₄), and evaporated. FC (400 g SiO₂, EtOAc/hexane 1:5 → EtOAc/hexane 1:1) afforded 8.35 g (93 %) of 4. R_f (EtOAc/hexane 1:2) 0.20.

Allyl 2,3,4-tri-*O*-benzyl- α -D-glucopyranuronide tert.-butyl ester (5). A solution of 4 (8.35 g, 17.0 mmol) in DMF/CH₂Cl₂ 4:1 (45 ml) was added to a solution of CrO₃ (6.8 g, 6.8 mmol) in DMF/CH₂Cl₂ 4:1 (180 ml) and pyridine (11.0 ml, 142 mmol) which had been stirred vigorously for 30 min r.t. After addition of Ac₂O (13.0 ml, 11.8 mmol) and tert.-BuOH (34.0 ml, 362 mmol), the solution was stirred for 9 h at r.t., before MeOH (30 ml) was added. After 30 min., the mixture was concentrated to one quarter of its volume and diluted with Et₂O (250 ml). Filtration through Na₂SO₄ and SiO₂ (300 g), elution with Et₂O, evaporation and FC (330 g SiO₂, AcOEt/hexane 1:9) afforded 6.30 g (66 %) of 5. R_f (EtOAc/hexane 1:2) 0.63. ¹³C-NMR (50 MHz, CDCl₃): 27.94 (q, triple intensity); 68.69 (t); 71.44 (d); 73.43 (t); 75.89 (t); 79.51 (d); 79.73 (d); 81.45 (d); 82.12 (s); 96.78 (d); 118.83 (t); 127.66 - 128.50 (several d); 133.56 (d); 138.06 (s); 138.21 (s); 138.69 (s); 168.84 (s).

Allyl 2,3,4-tri-*O*-benzyl- α -D-glucopyranuronide (6). A solution of 5 (6.25 g, 11.1 mmol) in HCOOH (150 ml) was stirred at r.t. for 30 min. Evaporation yielded 5.60 g (99 %) of chromatographically pure 6. R_f - (EtOAc/hexane/HCOOH 1:1:trace) 0.47. ¹³C-NMR (75 MHz, CDCl₃): 68.88 (t); 69.86 (d); 73.40 (t); 75.33 (t); 75.06 (t); 79.14 (d); 79.26 (d); 81.42 (d); 96.13 (d); 118.90 (t); 127.77 - 128.55 (several d); 133.18 (d); 137.47 (s); 137.84 (s); 138.46 (s); 174.18 (s).

Allyl (5*R*)-5-acetoxy-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside (7). A stirred solution of 6 (5.60 g, 11.1 mmol) in C₆H₆ (50 ml) and pyridine (5 ml) was treated with Pb(OAc)₄ (16.80 g, ca. 32 mmol) under N₂ at 60 ° for 25 min. Filtration through SiO₂, elution with Et₂O, evaporation and FC (300 g SiO₂, AcOEt/hexane 1:6) afforded 4.1 g (71 %) of 7. R_f (EtOAc/hexane 1:4) 0.29. IR (CHCl₃): 3089w, 3067w, 3008w, 2933w, 2874w, 1759s, 1497w, 1455m, 1367m, 1248w, 1161m, 1070s, 1028s, 937w. ¹³C-NMR (50 MHz, CDCl₃): 21.16 (q); 68.78 (t); 73.67 (t); 75.47 (t); 76.33 (t); 79.50 (d); 80.54 (d); 81.37 (d); 90.18 (d); 95.35 (d); 119.09 (t); 128.05-128.84 (several d); 133.68 (d); 138.36 (s); 138.63 (s); 139.01 (s); 169.75 (s).

Allyl (5*S*)-5-hydroxy-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside (**8**). At -78°, DIBAH (2.8 ml of a 20 % solution in toluene, ca. 2.9 mmol) was added dropwise to a solution of **7** (553 mg, 0.96 mmol) in CH₂Cl₂ (20 ml). After 15 min., a sat. solution of NH₄Cl (2 ml) was added. The mixture was warmed up to r.t., diluted with H₂O and a 1 M solution of H₂SO₄ (10 ml). The aq. layer was extracted with CH₂Cl₂ (3 x), the combined
 5 org. layers were extracted with brine (2 x), dried (MgSO₄) and evaporated to yield 499 mg (98 %) of crystalline **8** which was used without further purification in the next step. R_f (EtOAc/hexane 1:2) 0.32. ¹H-NMR (300 MHz, CDCl₃): 2.92 (broad s, OH); 3.33 (dd, J = 9.2, 7.8, H-C(4)); 3.60 (dd, J = 9.7, 3.7, H-C(2)); 3.99 (t, J = 9.5, H-C(3)); 4.07 (ddt, J = 12.9, 6.6, 1.2, OAlI); 4.23 (ddt, J = 12.9, 5.2, 1.4, OAlI); 4.65 (d, J = 12.0), 4.79 (d, J = 12.0, PhCH₂); 4.78 (d, J = 3.7, H-C(1)); 4.81 (d, J = 11.2), 4.89 (d, J = 11.9,
 10 PhCH₂); 4.85 (d, J = 10.9), 4.93 (d, J = 10.9, PhCH₂); 5.06 (d, J = 7.8, H-C(5)); 5.24 (dq, J = 10.3, 1.5, OAlI); 5.34 (dq, J = 17.2, 1.5, OAlI); 5.93 (dddd, J = 17.1, 10.3, 6.6, 5.2, OAlI); 7.28-7.42 (m, 15 arom. H).

Allyl (5*S*)-5-trichloroacetimidoyloxy-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside (**9**). MTBD (66 μ l, 0.46 mmol) was added to a cooled (-30°) solution of crude **8** (200 mg, ca. 0.42 mmol) and Cl₃CCN (0.63 ml, 6.3 mmol) in dry ClCH₂CH₂Cl (6 ml). After 10 min, the solution was filtered through SiO₂, the SiO₂ was eluted with
 15 Et₂O, and the combined filtrates were evaporated to give crude **9** which was sufficiently pure (¹H-NMR, TLC) to be used in the next step. R_f (EtOAc/hexane 1:2) 0.53.

Allyl (5*R*)-5-dimethylphosphonyl-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside (**10**) and allyl (5*S*)-5-dimethylphosphonyl-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside (**11**). TMSOTf (83 μ l, 0.46 mmol) was added to a cooled (-17°) solution of crude **9** (350 mg) and P(OMe)₃ (240 μ l, 1.26 mmol) in dry MeCN (6 ml). The solution was
 20 warmed to 0°, kept at this temperature for 3 h, and filtered through SiO₂. The SiO₂ was eluted with Et₂O, and the combined filtrates were evaporated. The residue (396 mg) was subjected to FC (22 g SiO₂, EtOAc/hexane 1:1) to give a mixture of **10** and its (5*S*) isomer **11** (147 mg, 62 % from **7**). This mixture was further purified by HPLC (EtOAc/hexane 2:1) to give 49 mg (21 % from **7**) of **10** and 52 mg (22 % from **7**) of **11**. Data of **10**: R_f (EtOAc/hexane 1:1) 0.12. ¹H-NMR (500 MHz, CDCl₃): 3.55 (dd, J = 9.6, 3.6, H-C(2)); 3.69 (d, J = 10.8, OMe); 3.80 (d, J = 10.5, OMe), 3.81 (dt, J = 10.6, 8.8, H-C(4)); 3.99 (t, J = 9.1, H-C(3)); 4.00 (ddt, J = 12.8, 6.6, 1.2 OAlI); 4.05 (dd, J = 10.5, 9.8, H-C(5)); 4.16 (ddt, J = 12.8, 5.2, 1.4, OAlI); 4.62 (d, J = 12.1), 4.77 (d, J = 12.1, PhCH₂); 4.80 (d, J = 10.6), 4.89 (d, J = 10.3 PhCH₂); 4.81 (d, J = 4.7, H-C(2)); 4.83 (d, J = 11.2), 4.97 (d, J = 10.9, PhCH₂); 5.24 (dq, J = 10.3, 1.1, OAlI); 5.33 (dq, J = 17.2, 1.6, OAlI); 5.93 (dddd, J = 17.1, 10.3, 6.7, 5.2, OAlI); 7.24-7.35 (m, 15 arom. H). ¹³C-NMR (125 MHz, CDCl₃): 52.74 (dq, J(P,C) = 6.8); 53.88 (dq, J(P,C) = 6.5); 65.86 (dd, J(C,P) = 175.1); 68.73 (t); 73.46 (t); 75.26 (t); 75.90 (t); 78.40 (dd, J(C,P) = 2.7); 79.35 (dd, J(C,P) = 1.0); 82.01 (dd, J(C,P) = 17.9; 96.40 (dd, J(C,P) = 15.0); 118.66 (t); 127.63-128.49 (several d); 133.28 (d); 138.00 (s); 138.11 (s); 138.63 (s). ³¹P-NMR (203 MHz, CDCl₃): 24.60.

Prop-1-enyl (5*R*)-5-dimethylphosphonyl-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside (**12**). A solution of activated 1,5-cyclooctadiene-bis[methyldiphenylphosphine]-iridium hexafluorophosphate (15 mg) in dry THF (5 ml) was added to a stirred solution of **10** (257 mg; 0.452 mmol) in dry THF (10 ml). After 2 h, TLC indicated completion of the reaction, and the solution was evaporated to give 57 mg of crude **12** which was used without purification in the next step. R_f (EtOAc/hexane 3:1) 0.39.

(5*R*)-5-dimethylphosphonyl-2,3,4-tri-*O*-benzyl- α -D-xylopyranose (**13**). A stirred solution of crude **12** (57 mg) and yellow HgO (118 mg; 0.54 mmol) in H₂O/acetone 1:10 (10 ml) was treated with a solution of HgCl₂ (148 mg; 0.55 mmol) in H₂O/acetone 1:10 (5 ml). After completion of the reaction, Et₂O was added. The Et₂O layer was washed with a semisaturated solution of KI and with brine. SiO₂ (2 g) was added, the mixture was evaporated and subjected to FC (15 g SiO₂, EtOAc/hexane 3:1 → EtOAc/hexane 5:1) to give 216 mg (90 % from **10**) of **13**. R_f (EtOAc/hexane 3:1) 0.16.

O-[(5*R*)-5-dimethylphosphonyl-2,3,4-tri-*O*-benzyl- α -D-xylopyranoside]-trichloroacetimidate(**14**). At -30°, MTBD (1.1 Eq.) was added to a solution (0.05 M) of **13** and Cl₃CCN (15 Eq.) in dry CH₂Cl₂. After completion of the reaction, the solution was filtered through SiO₂, the SiO₂ was eluted with Et₂O, and the combined eluants were evaporated to give crude **14** which was used without purification in the next step.

4-Methylumbelliferyl (5*R*)-5-dimethylphosphonyl-2,3,4-tri-*O*-benzyl- β -D-xylopyranoside (**15**). A solution of crude **14** (1 eq.) and 4-methylumbelliferone (2 Eq.) in dry MeCN (0.05 M) at -20° was treated with BF₃·OEt₂ (1 Eq.). After completion of the reaction, H₂O was added. The aq. phase was extracted with EtOAc (3x), the combined org. phases were washed with brine, dried over MgSO₄ and evaporated. The residue was subjected to FC to give **15**.

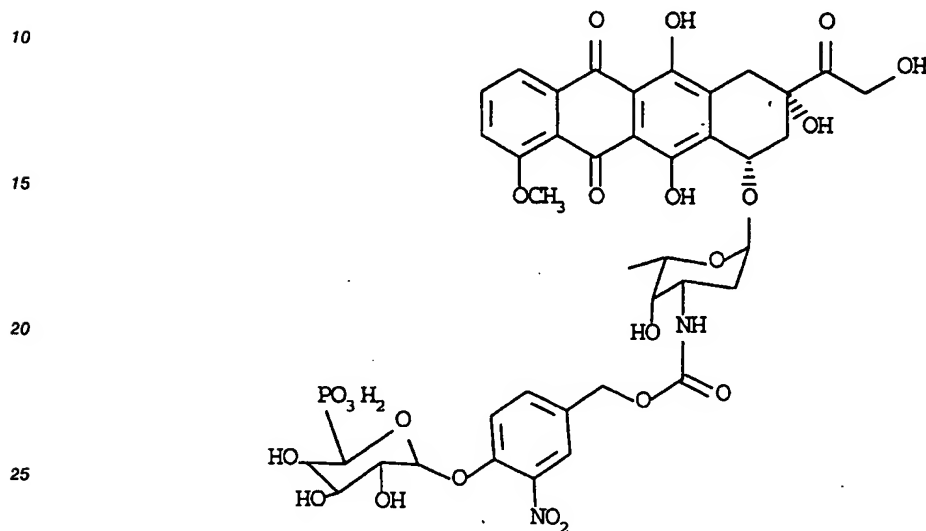
4-Methylumbelliferyl (5*R*)-5-dimethylphosphonyl- β -D-xylopyranoside (**16**). A solution of **15** in MeOH (0.05 M) was treated with H₂ in the presence of Pd/C 1:10 [K. Wallimann, Helv. Chim. Acta 1990, 73, 1359]. Filtration through Celite and evaporation gave crude **16** which was purified by FC (MeOH/EtOAc).

4-Methylumbelliferyl (5*R*)-5-phosphonyl- β -D-xylopyranoside (**17**). A solution of **16** in CH₂Cl₂ (0.05 M) was treated under N₂ at 0° with Me₃SiBr (30 Eq.) [C.E. McKenna, Tetrahedron Lett. 1977, 155]. After

completion of the reaction, MeOH was added, the mixture concentrated i.v., the residue taken up in H₂O, and the mixture lyophilized. Purification of the residue by anion-exchange chromatography (Dowex 1 x 8 (HCOO⁻): 0-0.7 M HCOOH) [K. Wallimann, *Helv. Chim. Acta* 1990, 73, 1359] gave 17 which was immediately transformed into its Na-salt by anion-exchange chromatography (Dowex 50 W x 4 (Na⁺)).

5 Prodrug D was synthesized analogously as described in WO 92/19639.

Prodrug D:



30 Example 3:

Comparison of Km- and Vmax-values of natural and improved substrate for antibody β -glucuronidase fusion protein

35 For Km- and Vmax-determination **3'-N-[4-(beta-D-Glucuronyloxy)-3-nitro-benzyloxycarbonyl]-doxorubicin and prodrug A** should be diluted in the range of 10-10000 μ M in 100 mM phosphate buffer + 1 mg/ml BSA, pH 7.2. Enzymatic cleavage should be done with constant amounts of fusion protein at 37 ° C. Cleavage can be monitored by HPLC analysis. Km- and Vmax-values can be calculated with the software program GraFit 2.0 (Erithacus Software Ltd.).

40

HPLC Analysis:

The HPLC apparatus consisted of an autosampler (Abimed, model 231), an automatic sample extraction system (AASP, Varian) equipped with minicartridges containing C 18 reversed phase silica gel (Analytichem), a gradient pump (Gynkotek, model 480), a fluorescence detector (Shimadzu RF 535, Excitation: 495 nm, Emission: 560 nm). Before sample injection the minicartridges were preconditioned with 2.5 ml methanol and 1.5 ml phosphate buffer, pH 6. Analytes retained on the reversed phase silica gel were then eluted by valve switching and connection of the minicartridges to the mobile phase. Chromatography was performed on reversed phase material (Nucleosil C 18, 5 μ m particle size, 120 mm length, 4,5 mm I.D.) and gradient elution. Elution was done by a gradient composed of 2 components (A: 20 mM phosphate, pH 3, B: acetonitrile). The gradient was run with the following time-concentration profile:

55

0 min:	75 % A, 25 % B
20 min:	25 % A, 75 % B
30 min:	25 % A, 75 % B

Before starting the next run the column was allowed to equilibrate at starting conditions for 5 min.

enzyme	substrate	K _m mM	V _{max} nmol/μg/min
antibody-β-glucuronidase fusion protein	3'-N-[4-(β-D-glucuronyloxy)-3-nitro-benzoyloxycarbonyl]-doxorubicin (glucuronide prodrug)	1.3	0.635
	prodrug A (K _m -reduced prodrug)	< 0.5	~ 0.635

Example 4:

Prodrug A can be encapsulated according to D. Papahadjopoulos et al. (PNAS, USA 88:11460-11464, 1991) into stealth liposomes. After i.v. injection into CD1 nu/nu mice the plasma clearance of Prodrug A encapsulated into stealth liposomes should be prolonged from ≈ 20 min for the free Prodrug A to ≈ 40 hrs for the encapsulated Prodrug A. The significant t_{1/2β} prolongation leads to improved pharmacological efficacy.

Claims

- Compound according to formula I,

S-Z-W (I)

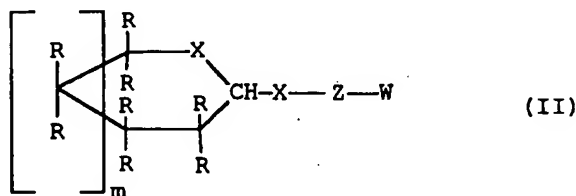
wherein

W means a pharmacologically active substance,

Z stands for a self-immolative spacer or a bond and

S is a modified competitive enzyme inhibitor such that the Z-S bond can be enzymatically cleaved at an at least 2-fold lower Michaelis Menten constant compared to the natural enzyme substrate.

- Compound according to claim 1 wherein the bond between S and Z is a glycosidic bond cleavable by an enzyme or a catalytic antibody.
- Compound according to claim 2 wherein the bond is cleavable by a human glycosidase, preferentially human β-glucuronidase.
- Compound according to claim 2 with the formula II,



wherein

R may be independent from each other H, OH, F, NH₂, COOH, CH₂-COOH, CHOH-COOH, PO₃H₂, CH₂-PO₃H₂ or CHOH-PO₃H₂,

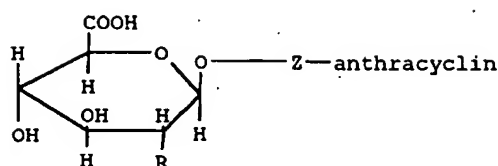
X may be NH, O or S,

m may be 0 or 1,

W means a pharmacologically active substance and

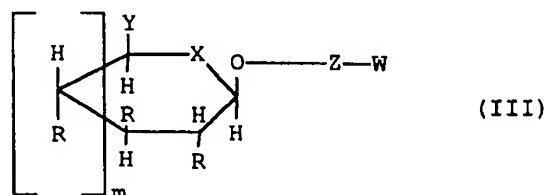
Z stands for a self-immolative spacer or a bond.

Not included are β-D-glucuronide-Z-anthracyclin compounds:



with R = OH, NH₂.

5. Compound according to claim 4 with the formula III,



wherein

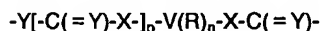
Y may be COOH, CH₂-COOH, CHOH-COOH, PO₃H₂, CH₂PO₃H₂ or CHOH-PO₃H₂,

X may be NH, O or S,

R may be independent from each other F, NH₂, H or OH,

m may be 0 or 1,

Z stands for a bond or a self-immolative spacer preferentially a moiety with the formula



wherein

V is an aromate or a hetero aromate or an aliphate with conjugated double bonds or an amino acid residue which cycles after cleavage of the glycosyl residue, preferentially with 5-20 carbon atoms and 0-4 hetero atoms, wherein hetero atom means N, O or S, substituted with being independently from each other H, methyl, methoxy, carboxy, methoxycarbonyl, CN, hydroxy, nitro, fluor, chlor, brom, sulfonyl, sulfonamid or sulfon (C₁₋₄)-alkylamid and

p 0 or 1

n an integer of 0 to 25, preferentially 1 or 2

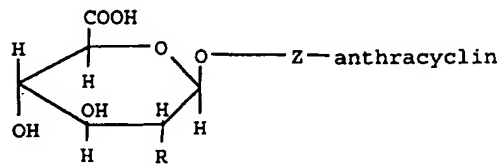
X O, NH, methylenoxy, methylenamino or methylen (C₁₋₄)-alkylamino,

Y O or NH and

W means a pharmacologically active substance, preferentially an anthracycline such as doxorubicin, 4'-epi-doxorubicin, 4- or 4'-desoxy-doxorubicin, or an etoposide, N-bis-(2-chlorethyl)-4-hydroxyaniline, 4-hydroxycyclophosphamide, vindesine, vinblastine, vincristine, terfenadine, terbutaline, fenoterol, salbutamol, muscarine, oxyphenbutazone, salicylic acid, p-aminosalicylic acid, 5-fluorouracil, 5-fluorocytidine, 5-fluorouridine, methotrexate, diclofenac, flufenamic acid, 4-methylaminophenazone, theophylline, nifedipine, mitomycin C, mitoxantrone, camptothecin, m-AMSA, taxol, nocodaxol, colchicine, cyclophosphamide, rachelmycin, cisplatin, melphalan, bleomycin, nitrogen-mustard, phosphoramidate-mustard, quercetin, genistein, erbstatin, tyrphostin, rohitukine-derivative ((-)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-4H-1-benzopyran-4-on; EP 89119710.5), retinoic acid, butyric acid, phorbol ester, DMSO, aclacinomycin, progesterone, buserelin, tamoxifen, mifepristone, onapristone, N-(4-aminobutyl)-5-chloro-2-naphtalen-sulfonamide, pyridinyloxazol-2-one, quinoly, isoquinolyloxazolone-2-one, staurosporine, ethanolamine, verapamil, forskolin, 1,9-dideoxyforskolin, quinine, quinidine, reserpine, 18-O-(3,5-dimethoxy-4-hydroxybenzoyl)-reserpate, lonidamine, buthionine sulfoximine, diethyldithiocarbamate, cyclosporine A, azathioprine, chlorambucil, N-(4-trifluormethyl)-phenyl-2-cyano-3-hydroxy-croton-acid-amide (WO 91/17748), 15-deoxyspergualin, FK 506, ibuprofen, indomethacin, aspirin, sulfasalazine, penicillamine, chloroquine, dexamethasone, prednisolone, lidocaine, propafenone, procaine, mefenamic acid, paracetamol, 4-aminophenazone, muskosine, orciprenaline, isoprenaline,

amiloride, p-nitrophenylguanidinobenzoate or their derivatives additionally substituted with one or more hydroxy-, amino- or iminogroups, linked through a hydroxy-, amino- or imino group to Z.

Not included are β -D-glucuronide-Z-anthracyclin compounds:



with R = OH, NH₂.

6. A pharmaceutical containing a compound according to claim 1.
7. A pharmaceutical according to claim 5 encapsulated in liposomes.
8. A pharmaceutical according to claim 5 in combination with pretargeted enzymes, catalytic antibodies, immunotoxins or immunoconjugates.



European Patent
Office

PARTIAL EUROPEAN SEARCH REPORT

Application Number

which under Rule 45 of the European Patent Convention EP 94 11 3388
shall be considered, for the purposes of subsequent
proceedings, as the European search report

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
D,X, Y	EP-A-0 511 917 (LAB. HOECHST S.A.) * page 5, line 1 - line 50 * * page 6, line 25; claims * & WO-A-92 19639 ---	1-7	A61K47/48
X,Y	EP-A-0 501 215 (BEHRINGWERKE AG.) * example T * ---	1-7	
Y	WO-A-81 01145 (UNIVERSITY OF ILLINOIS FOUNDATION) * page 19; figure I * * page 21; table 2 * * page 34, line 23; claims * ---	1-7	
Y	WO-A-90 03188 (NEORX CO.) * page 19, line 27 - page 24, line 33 * ---	1-7	
A	EP-A-0 540 859 (BRISTOL-MYERS SQUIBB CO.) * claim 1 * ---	1-7	
Y	EP-A-0 441 218 (BEHRINGWERKE) * page 5; claims; figures I,II * -----	1-7	
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			A61K
INCOMPLETE SEARCH			
<p>The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims</p> <p>Claims searched completely : Claims searched incompletely : Claims not searched : Reason for the limitation of the search:</p> <p>see sheet C</p>			
Place of search		Date of completion of the search	Examiner
THE HAGUE		28 November 1994	Berte, M
CATEGORY OF CITED DOCUMENTS			
<p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document</p>			

EPO FORM 1503 03.92 (P04C07)

BEST AVAILABLE COPY



EP 94 11 3388

-C-

INCOMPLETE SEARCH

Claims searched incompletely : 1-7

Reason : In view of the large number of compounds, which are designed by the general formulas of claim 1 and also in view of the definition of products by means of their biological, chemical and/or pharmacological properties, the search has to be restricted for economic reasons. The search was limited to the compounds for which pharmacological data was given and/or the compounds mentioned in the claims or examples (see Guidelines, Part B, Chapter III, paragraph 3.6)